Preliminary Observations of the Inking Behavior of *Aplysia (Varria)*

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As ONE STEP in the understanding of the phyletic development of emotional behavior, we have been investigating the responses of some invertebrates to noxious stimulation. The gastropod (opisthobranch) *Aplysia* will ink when handled roughly, when disturbed during copulation or when the mantle skirt is lifted, according to the literature (EALES, 1921). Others have said that after some experience, it is possible to always pick up, or "handle" the animal without causing it to ink. As "handling" seems to play some role in the elicitation of what is known as "emotional" behavior in rodents, we thought it would be interesting to study the response of *Aplysia* to handling. What are the stimuli which are most reliably followed by inking?

Two species were observed: 50 *Aplysia (Varria) dactylomela* RANG, 1828 and 3 *Aplysia (Varria) brasiliana* RANG, 1828 or *Aplysia floridensis* (PILSBRY, 1895).

Observations were made at low tide in the bay area adjoining the Lerner Marine Laboratory in Bimini, The Bahamas. Data were also gathered on animals in individual aquaria (8″x12″x6″) and large pens (3′x6′x2′), all equipped with drainage systems which permitted an efficient water circulation. Bay vegetation was provided to all animals as food.

The animals were manipulated by human hand in a standard fashion. The palm was placed over the visceral hump, parapodia and rear foot, the thumb and forefinger placed to the left and right of the head region, the remaining fingers curved under the foot of the animal. Between the manipulation of any two animals, the experimenters washed their hands and equipment. We will refer to this as "standard handling." Another form of mechanical stimulation was a series of sharp punches delivered in a consistent manner with a stick.

To identify individual animals, we found that a safety pin could be inserted through the parapodia with coded, colored time-tape attached to the pin. The pricking of the skin with the pin was followed frequently by inking, and so this was adopted as a standard form of intense stimulation. The punches and skin prick were termed "intense" stimulation.

In addition, electrical stimulation; drugs such as epinephrine, acetylcholine, prostygmine and urethane (see Table 1); and exposure to spiny sea-urchins, porcupine fish, various crabs, octopus, brittle stars and starfish were used to elicit inking. None of these were reliable.

Table 1

Inking Response to Various Drugs

Drugs	Concentration by volume	Number subjects	Animals inking
Epinephrine			
Experiment no. 1	10%	12	1
Experiment no. 2	10%	12	2
Acetylcholine	.004%	7	0
Prostygmine	.002%	6	0
Urethane	1%	9	3

Therefore the report we are making refers primarily to standard "handling" and "intense" stimulation as defined.

During collections in the field, intensely stimulated animals were more likely to ink than animals which were only handled (see Table 2).

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Table 2

A. Inking Response to Two Types of Stimulation in the Field

(number of animals)

	Intensely stimulated	Handled
Inking	14	8
Non-inking	4	13

χ^2 test: "p" = .02

B. Relationship Between Inking in the Field and Inking upon First Manipulation in the Laboratory

(number of animals)

Inking in Laboratory	Inking in Field: No	Inking in Field: Yes
Yes	6	6
No	14	13

McNemar test of change: "p" = .05

An analysis of field versus first laboratory inking showed that if an animal inked in the field it was not likely to ink when put into the pen or aquarium, upon arrival in the laboratory. But an analysis of the concomitance between field and inking in the laboratory at any time was not significant ("p" = .50).

We then began an inquiry into the factors which were concomitant with lack of inking. Why could we not uniformly elicit the inking response? Stimulation or handling in or out of water made no difference. An analysis of the time of day at which inking took place, revealed no trend. Neither was the interval between stimulations significant. However, when the data were organized in regard to the animal's being alone or with other species-mates immediately prior to stimulation, a relationship appeared which had not been clear before.

We found that when the animals were solitary in the field, inking was not differentially elicited by handling or increased stimulation. However, if they were in pairs, or larger groups, more intense stimulation would more reliably elicit inking than standard handling. Some aspects of the group situation seemed to be related to the inking response (see Table 3).

We then conducted an experiment in the laboratory with four groups of animals maintained as follows:

Group	from	to	to	no. of subjects
A.	group pen	indiv. aquar.	group pen	7
B.	indiv. aquar.	group pen	indiv. aquar.	7
C.	group pen	group pen	group pen	11
D.	indiv. aquar.	indiv. aquar.	indiv. aquar.	14

Table 3

Relation Between Inking and Grouping in the Field

(number of animals)

A. Animals which were not in groups ("solitary")

	Inking	Non-Inking
Intensely stimulated	9	4
Handled	5	8

Fisher's exact probability test: "p" not significant

B. Animals in pairs or groups ("grouped")

	Inking	Non-Inking
Intensely stimulated	5	0
Handled	3	5

Fisher's exact probability test: "p" = .05

Each animal was stuck with a pin upon being transferred. Our analysis first showed that the sequence of experimental maintenance (comparing groups A and B) made no difference. Combining these two groups, we found that if an animal had been in a group it was more likely to ink than if it had been alone. Groups C and D showed no change in likelihood of inking (see Table 4).

Table 4

Relationship Between Grouping and Inking in Laboratory

(number of animals)

A. Groups A and B combined

		Pen to Aquarium: Non-Inking	Pen to Aquarium: Inking
Aquarium to pen	Inking	0	3
	Non-Inking	3	8

McNemar test of change: "p" = .008

B. Group C

		First transfer from pen to pen: Non-Inking	First transfer from pen to pen: Inking
Second transfer from pen to pen	Inking	3	2
	Non-Inking	4	2

C. Group D

		First transfer from aquarium to aquarium: Non-Inking	First transfer from aquarium to aquarium: Inking
Second transfer from aquarium to aquarium	Inking	3	2
	Non-Inking	7	2

We then turned our attention to group behavior. These animals are hermaphroditic, protandrous, and non-self-fertilizing (Barnes, 1963; Fisher, 1870; Marcus &

Marcus, 1955; Marcus & Marcus, 1957; Morton, 1958; Robert, 1890; Si, 1931). During fertile copulations, as well as non-fertile copulations, one animal inserts the "penis" into the common genital aperture. The animal inserting the "penis" will be referred to as the "male." The receptive animal will be called the "female." The words "male" and "female" refer to posture and behavior, not to reproductive function. Most of the animals laid eggs while in the laboratory. In addition, only one animal out of 53 never showed copulatory behavior. Common, simple pairing of two animals, was seen as well as chains of three and four animals. In two instances reciprocal copulation of two animals, and in one instance reciprocal copulation of three animals also was noted. Although many of the animals assumed both the "male" and "female" role, as well as a simultaneous male and female position, there were indications that some animals tended to be dominantly male or female in behavior. In addition, some animals tended to pair predominantly with only certain other animals, as shown by a series of tests for copulatory behavior as well as data of animals in pens. There was no relationship between size of animal and copulatory role, although one very active "male" was among the smallest animals in the sample.

Although no relationship was found in the laboratory between level of copulatory behavior or role and inking, the report by Eales (1921) and our finding that grouped animals in the field are more likely to ink when intensely stimulated seem to indicate the possibility of a relationship between reproductive behavior, grouping and inking.

Some of the processes which could be involved in this relationship are first, facilitation (in the Sherringtonian sense) of ganglionic activity brought about by the activation of pathways as a result of tactile and chemical stimulation during coupling and chaining. The pedal, parietal and visceral ganglia innervate the implicated organs: parapodia, the purple and opaline glands in the pallial cavity, the common genital aperture into which the copulatory organ is inserted, as well as the genital tract.

Second, the stimulation of intensive interindividual contact or experimental disturbance of groups of animals may activate the opaline gland. This secretion is said to be toxic and may be irritating when in sufficient quantity. This in turn may lower the threshold of the ink gland. An artifactual, laboratory "crowding" effect does not seem to be likely in view of the field data.

Another hypothesis to be considered is that the release of the ink may be more related to intensity of stimulation facilitated by interindividual activity and reproductive state than to adaptive mechanisms involved in predator-prey relationships.

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